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**13. ABSTRACT** *(Maximum 200 Words)*

Breast cancer is the second most common cause of brain metastases, diagnosed in 10 to 15% of breast cancer patients and found at autopsy in 20 to 30%. Survival after detection of brain metastases can be short, and the therapy currently available only offers the hope of surviving one year to 20% of patients. Relatively little is known about how breast cancer cells metastasize to the brain, and what phenotypes characterize these cells. This is due in large part to the lack of suitable experimental models. The objective of the application is to develop experimental models to study the pathogenesis of breast cancer brain metastases, using intra-carotid artery injection of breast cancer cells into nude mice. This model will be used to test the hypothesis that brain metastases arise from the survival and growth of unique populations of cells. We found that a variant of the MDA-MB-231 breast cancer line, resulting from three cycles of injection and recovery of cells from brain metastases, has significantly greater potential for experimental metastasis in the brain of mice, following injection into the carotid artery. This variant will be used in continuing studies of the phenotype of breast cancer cells that metastasize to brain.

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**The Biology of Breast Cancer Brain Metastasis**

INTRODUCTION:

Breast cancer is the most common cancer of women in the United States of America. The prognosis for recurrence and death after treatment of the primary cancer is related to the disease stage and the presence of axillary lymph node metastases at the time of diagnosis. The majority of the deaths of women with breast cancer are the result of metastasis (1). In addition to the axillary lymph nodes, other sites where breast cancer metastases are found include the bone, liver, lungs and brain. Breast cancer is the second most common cause of brain metastases, after lung cancer. Brain metastases are diagnosed clinically in 10 to 15% of breast cancer patients, and found at autopsy in 20 to 30% (2). The survival after detection of brain metastases can be short, a matter of weeks, and the therapy currently available only offers the hope of surviving one year to 20% of patients (3). The median age of women developing breast cancer brain metastases is 51 years, which is several years younger than the age of women diagnosed with metastases to other organs. This may be a reflection of aggressive behavior of the breast cancers of relatively younger women. Another observation from reviews of clinical data is that the brain is not uncommonly the first site of relapse in patients who had previously received adjuvant systemic chemotherapy (3,4). This may be the result of the chemotherapeutic agents being unable to cross the blood-brain barrier and thus being ineffective against micrometastases. Experimental data from our previous studies suggest that the blood-brain barrier is intact around metastases of a certain size ($<0.2mm^2$), yet as the brain metastases enlarge the barrier becomes permeable (5). Although it is well known that breast cancers can metastasize to the brain, relatively little is known about how these metastases form, and what phenotypes are characteristic of breast cancers with brain-metastasizing potential. Without this information, the rational design of new therapies to prevent or control the growth of brain metastases is impossible. In large part, progress in understanding the biology of breast cancer brain metastasis has been limited by the lack of suitable cell lines and experimental models. The objective of this application is to develop experimental models to study the pathogenesis of breast cancer brain metastases, using intra-carotid artery injection of human breast cancer cells into nude mice. The repeated cycles of selection of cells from brain metastases and re-injection into mice will generate cell lines with enhanced brain-metastasizing potential. These will be compared with the original cells, and with variants from metastases in other organs of mice to determine the phenotypic characteristics of brain-metastasizing breast cancer cells. New therapeutic approaches need to be based upon the better understanding of the events involved in brain metastasis that may emerge from studies using this animal model.

BODY:

Objective: To isolate brain metastasizing variants of human breast cancer cells

a) MDA-MB-231 breast cancer cell line

*Methods:* The MDA-MB-231 human breast cancer cell line was transfected with an expression vector for enhanced green fluorescent protein (GFP), and a clone isolated with high expression of GFP (termed MDA-231 GFP). The cells were injected into the internal carotid artery of female nude mice ($2 \times 10^5$ cells) using the technique described by Schakert et al. (6). The mice were observed daily, and killed when moribund. The survival time of each mouse was recorded. The mice were observed under a fluorescent dissecting microscope fitted with GFP filters, to

illuminate the brain metastases and help in the dissection of tumor tissue from the brain parenchyma. Samples of the tumor tissues were finely minced and placed in culture flasks with medium containing 800 μg/ml G418, the selection antibiotic used to isolate the GFP-expressing cells. Cultures were established from the brain metastases from three mice per group, and the cells combined to create the *in vivo*-selected variant line. The cells were then injected into the carotid artery of female nude mice, and the recovery of cells and tissue repeated. The *in vivo* selection was repeated three times, resulting in variant cell lines named MDA-231 BR1, MDA-231 BR2 and MDA-231 BR3. Samples of tissue from the brains of mice injected with the different variants were fixed in formalin or frozen in OCT for preparation of cryostat sections.

*Immunohistochemistry*: Frozen sections of the brains with experimental metastases were hybridized with antibody against CD31/PECAM, to detect endothelial cells. Appropriate procedures for blocking endogenous peroxidase and non-specific hybridization were used. The sections were counterstained with hematoxylin. Sections of brain tissue from mice that were not injected with breast cancer cells were also stained, to assess the vessel density in the normal brain.

*Quantitation of CD31-positive vessels*: Ten random 0.159 $mm^2$ fields at x100 magnification were captured for each stained section (no hematoxylin counterstain on the imaged sections) using Optimas Image Analysis software (Bioscan, Edmond, WA). A structure was classified as a vessel on the basis of the criteria of Weidner *et al.* (7). The CD31-positive vessels were counted using Scion Image Beta 4.02 Win software (Frederick, MD).

*Determination of VEGF protein in culture medium*: The supernatants from cultures of the breast cancer cells (2 x $10^5$ cells per 35 mm dish) were collected after 72 h incubation, and VEGF concentration measured in cell free fluids by ELISA (reagents from R&D Systems, Minneapolis, MN).

*Results:*

*Incidence of experimental brain metastases:* Table 1 shows the results of injecting MDA-231 GFP cells and the brain metastasis-selected variants into the internal carotid artery of nude mice, expressed as the incidence of mice with brain metastases and the mean survival time. The mice injected with the metastasis-selected cells had significantly shorter mean survival than the mice injected with the original cell line (compared using the Student's t-test).

**Table 1: Incidence of experimental brain metastases and survival time of mice injected with MDA-231 GFP and brain metastasis selected variants**

| Cell line | Incidence of brain metastases | Mean survival time (days) | p value |
|---|---|---|---|
| MDA-231 GFP | 82.4% | 59.1 ± 2 | |
| MDA-231 BR1 | 91.7% | 51 ± 2.2 | 0.01 |
| MDA-231 BR2 | 100% | 45.9± 6 | 0.004 |
| MDA-231 BR3 | 100% | 41 ± 3.7 | 0.001 |

When the growth rates of the different cell lines shown in Table 1 were compared *in vitro*, there were no differences in the doubling times, which were all approximately 27 h when the cells are grown in medium with 5% fetal bovine serum.

Visualization of the brain metastases of the MDA-231 GFP was facilitated by expression of the fluorescent protein. However, the expression was significantly reduced in the cells that had be successively cycled through the mice. Less than 5% of the MDA-231 BR2 and BR3 cells were found to express GFP, using flow cytometry to detect the fluorescence, although >96% of the original MDA-231 GFP cells were brightly fluorescent.

*Counting microvessels in brain parenchyma and metastases*: The growth of tumors has been shown to be dependent on the development of adequate blood supply through angiogenesis (7). The vascularization of the breast cancer brain metastases was assessed by counting the numbers of CD-31 positive stained vessels in sections with tumor lesions. The average number of vessels / 0.159mm$^2$ field at 100 x magnification was 40.5 $\pm$ 4 in sections with brain metastases of MDA-231 GFP, while in normal brain sections there were 24.8 $\pm$ 3.9 vessels /0.159mm$^2$ at this magnification (p = 0.045, Student's t-test). Qualitatively, the size of vessels in the brain metastases was greater than those in normal brain (Fig. 1, Appendix).

*Measurement of VEGF in culture supernatants*: VEGF is an important angiogenic factor for breast cancer, and also promotes permeability of vessels. The variants selected from the brain metastases of MDA-231 GFP released significantly more VEGF into culture supernatants than the original cell line p= 0.036 (MDA-231 BR1), p= 0.002 (MDA-231 BR2), p=0.002 (MDA-231 BR3; Student's t-test). (Fig 2).

**Figure 2**

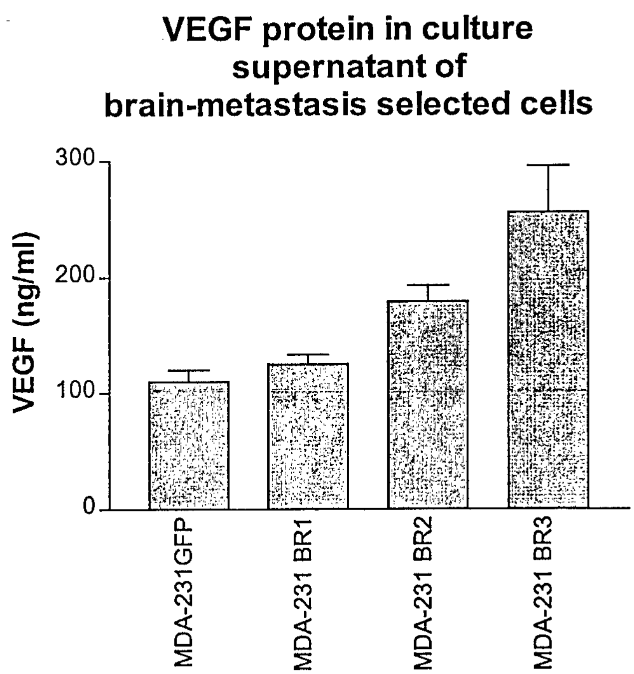


Fig 2: Culture supernatant was collected after 72 h incubation of 2 x 10$^5$ cells per 35 mm culture dish. VEGF content in the cell-free fluid was measured using ELISA (with reagents from R&D Systems to detect VEGF-165 isoform). Values were adjusted to reflect ng VEGF per ml of culture medium, and values shown are the mean and SD from triplicate samples.

b) MDA-MB-435 breast cancer cell line

*Methods*: As described above, the MDA-MB-435 cells were injected into the carotid artery of female nude mice, and the incidence of experimental brain metastases and survival times of the mice were recorded. Samples of tumor were dissected from the brains of three mice, and cultures established. These were pooled to derive the cell line MDA-435 KBR1. Additional variants of the MDA-MB-435 cell line used in comparison were: variants previously isolated from a brain metastasis (MDA-435 Br1) and lung metastases (MDA-435 Lung2) in a mouse with a mammary fatpad tumor (8); a line termed MDA-435 LVBr1, from a brain metastasis in a mouse injected via the left ventricle of the heart (Price, unpublished).

*Determination of VEGF protein in culture medium*: The supernatants from cultures of the breast cancer cells (2 x $10^5$ cells per 35 mm dish) were collected after 72 h incubation, and VEGF concentration measured in cell free fluids by ELISA (reagents from R&D Systems, Minneapolis, MN).

*Results*: Injection of the MDA-MB-435 cells into the carotid artery of mice resulted in brain metastases in 83% of mice, and mean survival time of 82 days. The line derived from brain metastases, MDA-435 KBR1 was injected into mice via the carotid artery, resulting in brain metastases in 86% of mice with a mean survival time of 54 days.

*Measurement of VEGF in culture supernatants*: Fig 3 shows a comparison of the levels of VEGF-165 in the supernatant of the different variants of MDA-MB-435, isolated from brain metastases or lung metastases. Significantly more VEGF was released from two of the brain metastases derived variants than the original line, or the lung metastasis derived variant. Repeat assays gave similar results, with the brain metastasis derived variants releasing more VEGF.

Figure 3

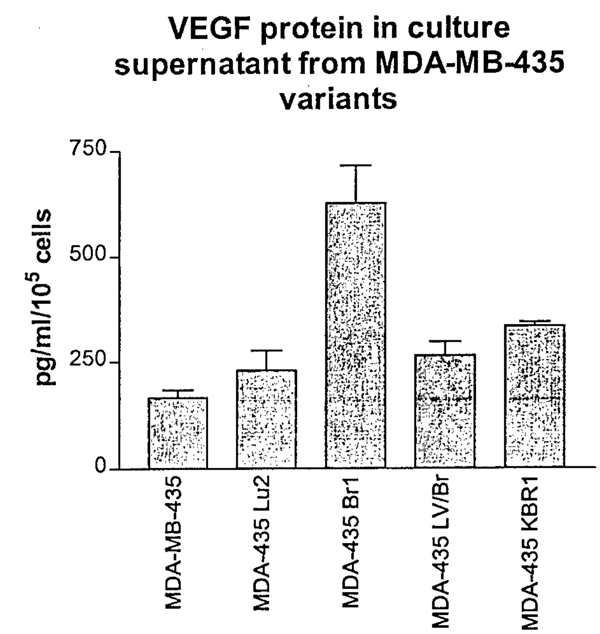


Fig. 3: Culture supernatant was collected after 48 h incubation of 2.5 x $10^5$ cells per 35 mm culture dish. The VEGF content in the cell free-fluid was measured using ELISA (with reagents from R&D Systems to detect VEGF-165 isoform). Values were adjusted to reflect ng/ml/$10^5$ cells, and mean and SD from duplicate cultures are shown in the figure. Supernatants from the brain metastasis-derived cells had higher concentrations of VEGF than the original cell line or the lung metastasis derived line: MDA-435-Lu2, p= 0.2; MDA-435-Br1, p= 0.019; MDA-435LV/Br, p=0.06, MDA-435KBR1, p=0.007, each compared with value for MDA-MB-435, Student's t-test.

*Ongoing studies*: The variants of the breast cancer cell lines isolated from brain metastases are currently being used in continuing phenotypic characterizations, as in the original Statement of

Work. These were: a) Injecting cells by other routes into mice, to assess tumorigenicity and metastatic capacities to organs other than the brain: b) Measuring mRNA and/or protein expression of proteolytic enzymes, growth factor receptors, cytokines and cytokine receptors, comparing the original and brain metastasis-derived variants. The studies will also use variants of the cell lines that have been derived from metastases in other organs of mice, to test whether altered gene expression is a function of the organ site of the metastasis, or of selection from metastases in general.

KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of variants of human breast cancer cell lines from experimental brain metastases in nude mice, following intra-carotid artery injection of the cells.
- Demonstration that the brain metastasis-selected cell lines have enhanced ability to produce brain metastases in nude mice, compared with the original cell lines.
- Demonstration that the brain metastasis-selected cells have elevated expression of the angiogenic and permeability inducing factor VEGF.

REPORTABLE OUTCOME:

Abstract in the Proceeding of the American Association for Cancer Research (see Appendix) and presentation in a poster session at the Annual Meeting of the AACR, March 2001, "A Model of Human Breast Cancer Brain Metastasis", L.S. Kim and J.E. Price.

CONCLUSIONS:

The hypothesis being tested in this project is that breast cancer cells in brain metastases represent specialized populations of cells endowed with the ability to survive and grow in the brain. The initial results from the study using a nude mouse model of experimental brain metastases to isolate selected populations suggest that these do differ from the original cell lines. The brain metastasis-derived cells have enhanced potential for metastasis in the brain. In addition, these selected cells show higher expression of VEGF, a factor that promotes angiogenesis and the permeability of blood vessels. Significant points of these results are: 1) The selected cell lines can be used to identify phenotypes that may be unique to, or over-expressed in breast cancer cells with the ability to survive and grow in the brain. 2) The breast cancer cell lines injected into the carotid artery of nude mice is a reliable model of experimental brain metastases, which can be used for future studies testing novel approaches of therapy.

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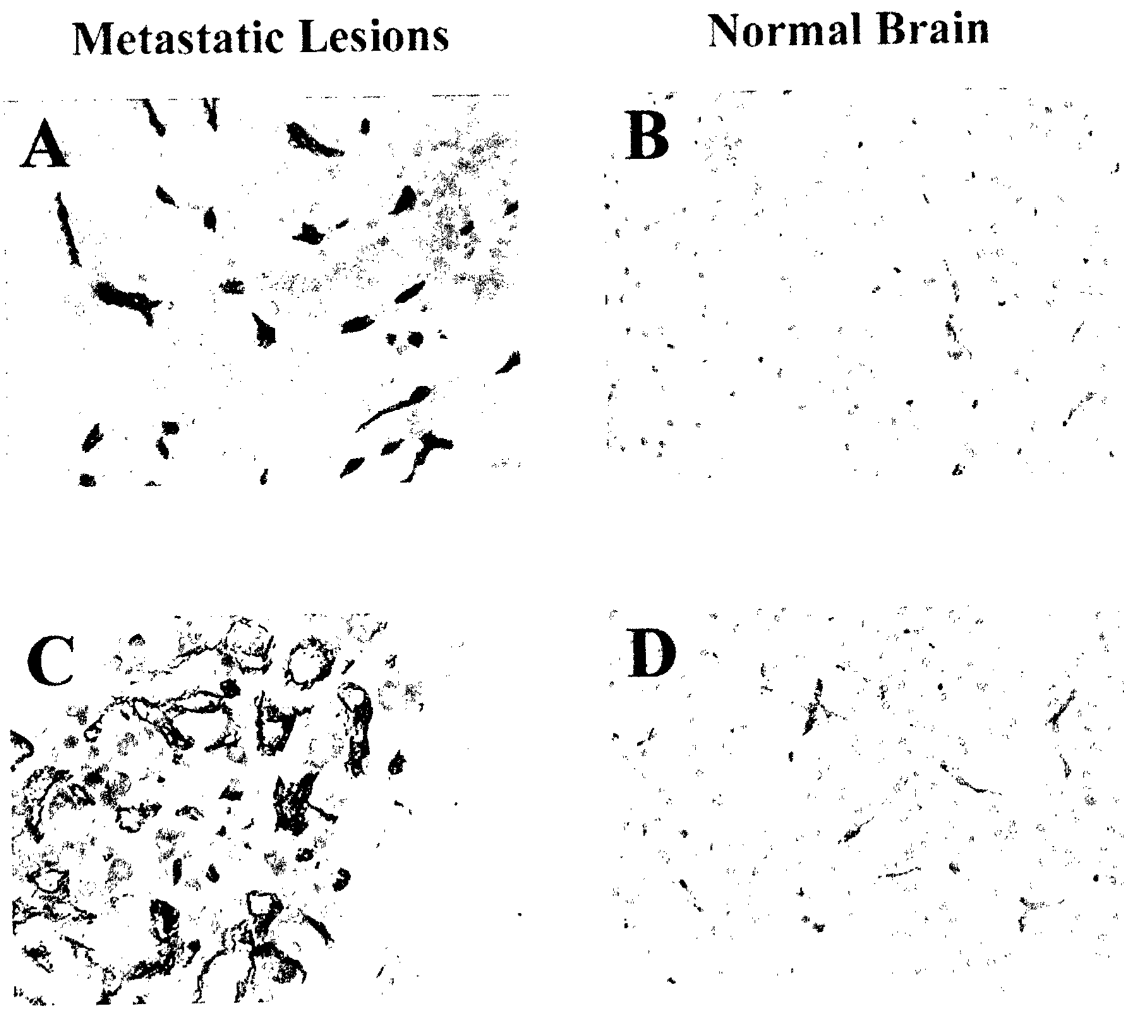
## Figure 1: CD31-positive vessels in brain metastases and uninvolved brain tissue




**CD-31 positive blood vessels in experimental brain metastases (panels A and C) and in uninvolved brain (panels B and D). The density and size of the vessels were greater in the tumor lesions then in the normal brain tissue.**

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# A Model of Human Breast Cancer Brain Metastasis

**Lee Su Kim, Janet E. Price, UT M.D. Anderson Cancer Center, Houston, TX.**

Breast cancer is the second most common cause of brain metastases, after lung cancer. However, relatively little is known about how the metastases form, and what phenotypes are characteristic of brain-metastasizing breast cancer cells. The objective of the study is to develop reliable models, using human breast cancer cell lines injected into nude mice, with which to study the biology of brain metastasis growth. Three human cell lines were injected into the internal carotid artery of nude mice, using the technique described by Schackert et al in this department. Following injection of 1 x 105 cells in a volume of 0.1 ml, the incidence of brain metastasis and mean survival time of the mice injected with the different cell lines was as follows: MDA-MB-435, 83.3%, 81.6 days; MDA-231GFP (cells transfected with GFP gene), 76.9%, 62.4 days; GI101LM1b, 80%, 61.3 days. All three cell lines formed metastases in the brain parenchyma. In some mice injected with MDA-231GFP, the most aggressive cell line, the metastases invaded through the skull. The expression of green fluorescent protein (GFP) by the MDA-231GFP cells was used as a marker to aid in the dissection of the metastatic cells from the parenchyma of the mouse brain. Cells recovered from brain metastases were expanded in culture, and the cells re-injected into the mice. The incidence of brain metastasis in mice injected with the selected cell line MDA-231BR1mix was higher (87.5%) and survival time was significantly shorter (mean of 52.6 days, p=0.01, Student's t-test) than the survival of mice injected with MDA-231GFP cells, suggesting this is a population with enhanced potential for metastasis in the brain. The microenvironment of the brain of mice appears to be a favorable site for the growth of metastatic human breast cancer cells. The model can be used to analyze the phenotype of cells with potential for growth in this organ, and for testing novel therapies.

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